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Interleukin-4- and NACHT, LRR and PYD domains-containing protein 3-independent mechanisms of alum enhanced T helper type 2 responses on basophils

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Summary

Aluminium hydroxide (alum), the most widely used adjuvant in human and animal vaccines, has long been known to promote T helper type 2 (Th2) responses and Th2-associated humoral responses, but the mechanisms have remained poorly understood. In this study, we explored whether alum is able to directly modulate antigen-presenting cells to enhance their potency for Th2 polarization. We found that alum treatment of dendritic cells failed to show any Th2-promoting activities. In contrast, alum was able to enhance the capacity of basophils to induce Th2 cells. When basophils from interleukin-4 (IL-4) knockout mice were examined, the intrinsic Th2-promoting activities by basophils were largely abrogated, but the alum-enhanced Th2-promoting activities on basophils were still detectable. More importantly, Th2-promoting adjuvant activities by alum found in IL-4 knockout mice were also largely reduced when basophils were depleted by antibody administration. Therefore, basophils can mediate Th2-promoting activities by alum both *in vitro* and *in vivo* through IL-4-independent mechanisms. Further studies revealed that secreted soluble molecules from alum-treated basophils were able to confer the Th2-promoting activities, and neutralization of thymic stromal lymphopoietin or IL-25 attenuated the IL-4-independent development of Th2 cells elicited by alum-treated basophils. Finally, alum was able to activate NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome in murine basophils in the same way as alum in professional antigen-presenting cells, but NLRP3 was not required for Th2-promoting activities on basophils by alum *in vitro*. These results demonstrated that alum can enhance the capacities of basophils to polarize Th2 cells via IL-4- and NLRP3-independent pathways.

Keywords: aluminium hydroxide; basophils; T helper type 2 response; interleukin-4; NACHT, LRR and PYD domains-containing protein 3.

Introduction

Aluminium hydroxide and other aluminium salts, generally referred to as 'alum', have been widely used for decades as the major adjuvant to boost vaccine effects in humans.[1] Despite its routine use, the mechanisms by which alum enhances immune responses remain elusive. A commonly held view is that aluminium-adjuvanted vaccines form antigen depots from which antigens are slowly released to facilitate a long-lasting immune response.[2] However, it has been shown that antigens injected together with alum defuse very rapidly and are quickly cleared from the site of administration.[3] Indeed, the adjuvant effects by alum are not affected if injection sites are excised as early as 2 hr after injection, by which time antigens deposited at the local injection site are largely removed.[4] Therefore,

Abbreviations: a-basophils, alum-pretreated basophils; alum, aluminium hydroxide; APCs, antigen-presenting cells; BMDB, bone-marrow-derived basophils; BMDC, bone-marrow-derived dendritic cells; BMDMs, bone-marrow-derived macrophages; NLRs, nucleotide-binding oligomerization domain (NOD)-like receptors; NLRP3, NACHT, LRR and PYD domains-containing protein 3; TSLP, thymic stromal lymphopoietin

adjuvant effects of alum more likely result from quick stimulation of cellular events rather than being solely dependent upon depot effects. Alum is well known to greatly boost IgG1 and IgE responses against co-injected antigens, together with enhanced interleukin-4- (IL-4) or IL-5-producing T helper type 2 (Th2) cells.[5] Therefore, studies on mechanisms of alum adjuvanticity are reasonably centred on how alum induces Th2 differentiation.

Characterized by generation of IL-4, IL-5 and IL-13, Th2 cells are primarily polarized by IL-4.[6–8] Administration of alum is found to be able to promote Th2 differentiation with unclear mechanisms.[5] Being deficient for IL-4 or for the molecules required for mediating IL-4 responses failed to totally abrogate Th2 cells among mice being immunized with alum-adsorbed antigens.[9] Hence, IL-4 does not appear to be completely responsible for the induction of Th2 differentiation caused by alum, and IL-4-independent mechanisms should ascribe to some of the alum-induced Th2 activities. Furthermore, the IL-4-independent pathway may play important roles in the initiation of Th2 cells. No definitive cellular mechanisms have been reported on IL-4-independent Th2 promotion effects by alum. In this study, we investigated the cell types that can be directly affected by alum to promote Th2 differentiation via IL-4-independent mechanisms.

As the most important antigen-presenting cells (APCs) in activation and differentiation of naive $CD4^+$ T cells, dendritic cells (DCs) are shown to be critically required for induction of alum-induced Th2 responses *in vivo*.[10] However, whether or not alum can polarize DCs into Th2-promoting DC2 cells directly is not certain.[11,12] In our study, we failed to find that alum treatment of conventional DCs directly induces DC2 cells. Hence, cells other than conventional DCs may be involved in response to alum to allow Th2 differentiation.

A recent report has demonstrated that basophils are required for alum-promoted Th2 induction when haptens or peptide antigens are mixed with alum.[13] Accounting for < 1·0% of leucocytes in peripheral blood, basophils have been repeatedly recognized as APCs that are critical in initiation or progression of Th2-associated responses like anti-helminth immunity and allergic responses.[14–16] Basophils are naturally potent Th2-promoting APCs mainly due to their abilities to produce IL-4, by which basophils are found to promote Th2 differentiation either independently or via cooperation with DCs.[14–17] In this paper, we found that alum is able to directly enhance basophils but not DCs to induce Th2 differentiation *in vitro*. Furthermore, this enhanced Th2-promoting activity by alum on basophils is mainly mediated through an IL-4-independent pathway, which is also in action *in vivo*. Alum stimulates basophils to produce increased amounts of thymic stromal lymphopoietin (TSLP) and IL-25 transcripts independent of IL-4, and neutralization of these two Th2-promoting cytokines resulted in reduced development of Th2 cells elicited by alum-treated basophils. Finally, as in professional APCs, NACHT, LRR and PYD domains-containing protein 3 (NLRP3), the intracellular sentinel receptor known for sensing the presence of alum, is also expressed in basophils. However, NLRP3 is not required for the described Th2-promoting activities on basophils by alum. Our study provides evidence that basophils contribute to alum-promoted IL-4-independent Th2 polarization.

Materials and methods

Mice

Wild-type (WT) BALB/c mice and WT C57BL/6 (B6) mice were purchased from SLAC Laboratory (Shanghai, China). The IL-4-knockout mice (stock number: 002496) were purchased from Jackson Laboratories (Bar Harbor, ME); the DO11.10 OVA-TCR transgenic mice [C.Cg-Tg (DO11.10)10Dlo/JNju] were purchased from the Model Animal Research Centre of Nanjing University. Line of OT-II OVA-TCR transgenic mice [B6.Cg-Tg (TcraTcrb) 425Cbn/J] were kindly provided by Dr Chen Dong from the School of Life Science Tsinghua University. All mice were housed and bred under specific pathogen-free conditions in the Animal Centre of Tongji University and used at 6–8 weeks of age. All animal experiments were performed in accordance with guidelines of the Ethics of Animal Experiments Committee of Tongji University (Permit Number: 11050096).

Immunization and stimulation of lymphocytes

Wild-type (WT) BALB/c mice and IL-4-knockout (IL-4-KO) mice were immunized via the footpad with 25 μg ovalbumin (OVA) protein (Sigma, St Louis, MO) or 5 μg OVA peptide (OVA 323-339; Chinese Peptide Company, Hangzhou Economic and Technological Development Zone, China) adsorbed to 40 μl alum adjuvant (Sigma). Control mice were injected with OVA protein or OVA peptide in PBS. In some experiments, mice were intraperitoneally injected with 10 μg MAR-1 antibody (Biolegend, San Diego, CA), which is specific for FcεRIα 1 day before immunization for the depletion of basophils. Four days after immunization, draining lymph nodes were collected and cells were re-stimulated with plate-bound anti-mouse CD3ε (10 μg/ml; Biolegend) and soluble anti-mouse CD28 (2 μg/ml; Biolegend) for 48 hr, cell culture supernatants were collected for detection of IL-5. Depletion of basophils was monitored at days 1 and 4 in the blood by FACS.

Generation of bone-marrow-derived basophils, dendritic cells and macrophages

Bone marrow cells were flushed from the femurs and tibiae of 6- to 10-week-old WT, IL-4-KO, and NLRP3 knockout

(NLRP3-KO) mice. Isolated cells were cultured in RPMI-1640 medium (Gibco, Invitrogen, Grand Island, NY) or Dulbecco's modified Eagle's medium (Wisent Bioproducts, St Bruno, QC, Canada) containing 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen), 50 μM 2-mercaptoethanol (SERVA Electrophoresis GmbH, Heidelberg, Germany). The bone-marrow-derived basophils (BMDBs) were isolated based on the previously described method.[18] Briefly, after lysis of red blood cells, bone marrow cells were cultured at a cell density of 5×10^6/ml in the presence of 30 ng/ml IL-3 (Peprotech, Rocky Hill, NJ). Cells were replenished with IL-3-containing medium and replaced every 3–4 days to a cell density of 1×10^6/ml. The cells were harvested after 11 or 12 days in culture and stained with allophycocyanin-labelled anti-mouse FcεRIα (MAR-1), phycoerythrin-labelled anti-mouse c-kit (2B8), FITC-labelled anti-mouse CD49b (DX5) and Peridinin chlorophyll protein-Cy5.5-labelled anti-mouse CD11c (N418). Basophils ($FcεRIα^+$ $CD49b^+$ $c\text{-}kit^-$ $CD11c^-$) were sorted using FACSAria II (BD, Biosciences, San Jose, CA) in yielding purity of > 95%.

For the generation of bone-marrow-derived DCs (BMDCs), the bone marrow cells were cultured in the presence of 25 ng/ml granulocyte–macrophage colony-stimulating factor (Peprotech) and 1 ng/ml IL-4 (Peprotech). Five days later, non-adhered or loosely adhered cells were harvested and purified using biotinylated anti-mouse CD11c (N418; Biolegend) plus avidin magnetic beads (BD). Then, isolated DCs were detected by staining with phycoerythrin-labelled anti-mouse CD11c (N418) using FACSVerse (BD) and the purity was > 95%. Flow cytometry data analysis was analysed by FlowJo software (Treestar, Ashland, OR).

Bone-marrow-derived macrophage (BMDMs) were obtained from adhered population after bone marrow cells were cultured in Dulbecco's modified Eagle's medium supplemented with 30% conditioned medium from L929 cells (kindly provided by Dr Xin Lin from the School of Life Science, Tsinghua University) expressing macrophage colony-stimulating factor and 10% fetal bovine serum. Usually more than 95% adhered cells were positive for F4/80 (BM8) after 1 week in culture. All the staining antibodies above were bought from Biolegend.

Co-culture of naive $CD4^+$ T cells and APCs

Naive splenic $CD4^+$ T cells were sorted from DO11.10 mice using Peridinin chlorophyll protein-Cy5.5-labelled anti-mouse CD4 (GK1.5), phycoerythrin-labelled anti-mouse CD62L (MEL-14), FITC-labelled anti-mouse TCR DO11.10 (KJ1-26) or from OT-II mice using FITC-labelled anti-mouse Vα2 TCR (B20.1) instead of KJ1-26. The purity of the sorted naive $CD4^+$ T cells was > 99%. The above staining antibodies were purchased from eBioscience (San Diego, CA).

Naive $CD4^+$ T cells were plated at a density of 2×10^6/ml. These naive $CD4^+$ T cells were activated by APCs such as basophils (1×10^6/ml), DCs (4×10^5/ml) or a combination of basophils (1×10^6/ml) and DCs (4×10^5/ml) in round-bottomed 96-well plates containing 0·2 ml RPMI-1640 complete medium with OVA protein at 40 μg/ml (Sigma) or OVA (323–339) (Chinese Peptide Company) at 5 μg/ml. On the third day, culture medium was replaced by fresh complete medium containing 10 ng/ml recombinant human IL-2 (Peprotech) and cells were transferred into 48-well plates. After a further 2–3 days of expansion, cells were harvested and detected by intracellular cytokine staining as described below.

When alum (Sigma) treatment occurred, basophils were pretreated with alum for 30 min before $CD4^+$ T cells were added. Alum was used at dose of 50 μg/ml except for those noted. In some experiments, IL-4-KO basophils were stimulated by the indicated dose of alum for 18 hr. The supernatants were then collected and high-speed centrifuged to remove cells and added alum. Cell- and alum-free supernatants were added into the IL-4-KO basophils and DO11.10 $CD4^+$ T cells co-culturing system at half of the total culture volume. The supernatants from IL-4-KO basophils without alum stimulation were used as control. In neutralization experiments, neutralizing antibodies against IL-25 (514403; Biolegend) or TSLP (16-5491; eBioscience) or IL-25 plus TSLP or control Rat IgGs (Biolegend) were added into supernatants from alum-treated IL-4-KO basophils for 1 hr before supernatants were added.

Intracellular cytokine staining

Detection of intracellular cytokine expression was performed as described previously.[19] Briefly, culture cells were stimulated with PMA (20 ng/ml; Sigma) and ionomycin (1 μg/ml; Beyotime, Biotechnology, Shanghai, China) for 5 hr in the presence of brefeldin A (1 : 1000; BD) during the last 2 hr. T helper polarization profiles were determined with allophycocyanin-labelled anti-mouse IL-4 (11B11; Biolegend) for Th2 and phycoerythrin-labelled anti-mouse interferon-γ (XMG1.2; Biolegend) for Th1. Fluorophore- and isotype-matched antibodies against anti-cytokine antibodies were included. Fixable viability staining from eBioscience was added to exclude signals from dead cells. Then, intracellular cytokine signals were analysed after being gated on $CD4^+$ T cells.

In the experiment detecting cytokine production by splenocyte, spleen cells removed from OVA-immunized mice were stimulated with PMA (50 ng/ml), ionomycin (0·5 μg/ml) and brefeldin A (1 : 1000) together for 5 hr. Then intracellular IL-5 staining was performed by allophycocyanin-labelled anti-mouse IL-5 (TRFK5; Biolegend) and analysed after gating on $CD3^+$ $CD4^+$ spleen cells. Fixable viability staining from eBioscience was added to exclude signals from dead cells.

Proliferation assay

Naive splenic $CD4^+$ T cells from DO11.10 mice were labelled with CFSE (10 μM; Invitrogen) before being co-cultured with unstimulated or alum-treated basophils at 1 : 2 ratio in the presence of OVA (40 μg/ml) or OVA (323–339) (5 μg/ml). After 60 hr, cells were harvested and OVA-specific T-cell proliferation was determined on gated $CD4^+$ T cells.

Immunofluorescence

Lymph node frozen sections 10 μm in thickness were fixed in cold acetone for 10 min at 4° and stained with primary antibodies at 4° overnight. To stain basophils, fixed sections were incubated with rat anti-mMCP8 (TUG8; Biolegend), followed by biotinylated goat anti-rat IgG secondary antibody and Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories) or in some situations by FITC-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories). To stain DCs, primary antibody biotin-conjugated anti-CD11c (N418; Biolegend) was used, which was detected by Cy3-conjugated streptavidin. And to stain T cells, sections were stained with rabbit anti-mouse CD3 (GB13014; Wuhan Google Biological Technology, Wuhan, China), and then incubated with Dylight 488 conjugated with goat anti-rabbit IgG (GAR4882; Multi Sciences, Hangzhou, China). Nuclei were counterstained with DAPI. Slides were analysed on a Nikon eclipse 80i microscope (Nikon, Japan).

ELISA

Cell culture supernatants were assayed for IL-4 and IL-5 with ELISA kits from eBioscience according to the manufacturer's instructions.

RT-PCR analysis

Sorted WT or IL-4-KO basophils (1×10^6/ml) were treated with or without alum (50 μg/ml) for 4 hr, total RNA was extracted using RNAiso Plus (Takara). RNA was reverse transcribed using PrimeScript®RT reagent Kit (Takara, Shiga, Japan). The PCRs were performed with SYBR® Premix Ex TaqTM (Takara) with primers listed in the Supplementary material (Table S1) for IL-4, IL-25, TSLP, GATA-3 and GAPDH. Real-time PCR data were analysed using the $2^{-\Delta\Delta Ct}$ method in which GAPDH served as the endogenous gene. All reactions were run on ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Data were shown as averages from three biological repeats plus standard error.

For analysis of the expression of nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) mRNA, total RNA was extracted and reverse transcribed as described above. Specific NLR primer sequences are shown in the Supplementary material (Table S2). PCR conditions were as follows: cDNA was amplified for 35 cycles (except β-actin which was run for 25 cycles) of 94° for 30 seconds, 30 seconds at optimal annealing temperatures (see Supplementary material, Table S2) and 72° for 1 min with final extension of 72° for 5 min. After PCR, equal volumes of each PCR product were loaded onto 1% agarose gel containing ethidium bromide and visualized under UV light. Signals were semi-quantified by densitometry after being normalized by the density of β-actin.

Measurement of NLRP3 activation by alum

Sorted BMDBs from WT and NLRP3-KO mice were seeded on six-well plates at a cell density of 1×10^6/ml overnight. Cell-culture medium was replaced with opti-MEM medium (Gibco, Invitrogen) for stimulation. When cells were activated by alum, cells were primed with lipopolysaccharide (100 ng/ml; Sigma) for 3 hr first before they were treated with different doses of alum for another 6 hr. Cell-free supernatants were collected for detection of mature IL-1β p17 or cleaved caspase-1 p10 and cell lysates were collected for detection of pro-IL-1β, pro-caspase-1 and β-actin.

Cell lysates and supernatants were subjected to standard Western blotting analysis as described elsewhere.[20] Briefly, after stimulation with alum, proteins from cell-free supernatants were extracted by methanol/chloroform precipitation.[20] The protein pellet was dried at 55°, resuspended in Laemmli buffer, and boiled at 99° for 5 min, and stored at −80° until analysis. Whole cell lysates were made after cells were lysed in lysis buffer (Beyotime) supplemented with a protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA). The isolated protein samples from cell-free supernatants were separated on 15% SDS–PAGE or cell lysates on 10% SDS–PAGE and were transferred onto PVDF membranes. As indicated, blots were incubated with rabbit polyclonal antibody to anti-mouse caspase-1 p10 (SC-514; Santa Cruz Biotechnology, CA), rabbit polyclonal anti-mouse IL-1β (ab9722; Abcam, Hong Kong) or anti-mouse Actin antibody (M20010; Abmart, Shanghai, China). Anti-rabbit IgG-horseradish peroxidase (HRP) and anti-mouse IgG-HRP (Ding Guo, Beijing, China) were used as secondary antibodies. The final detection was made by adding chemiluminescent HRP substrate (Millipore, Billerica, MA) and read by chemiluminescence digital imaging system (ImageQuant LAS 4000 mini; GE Healthcare, Chalfont St Giles, UK).

Statistics

Statistical differences were analysed by analysis of variance or Student's *t*-test using GRAPHPAD PRISM software (GraphPad, San Diego, CA). Statistics were performed as

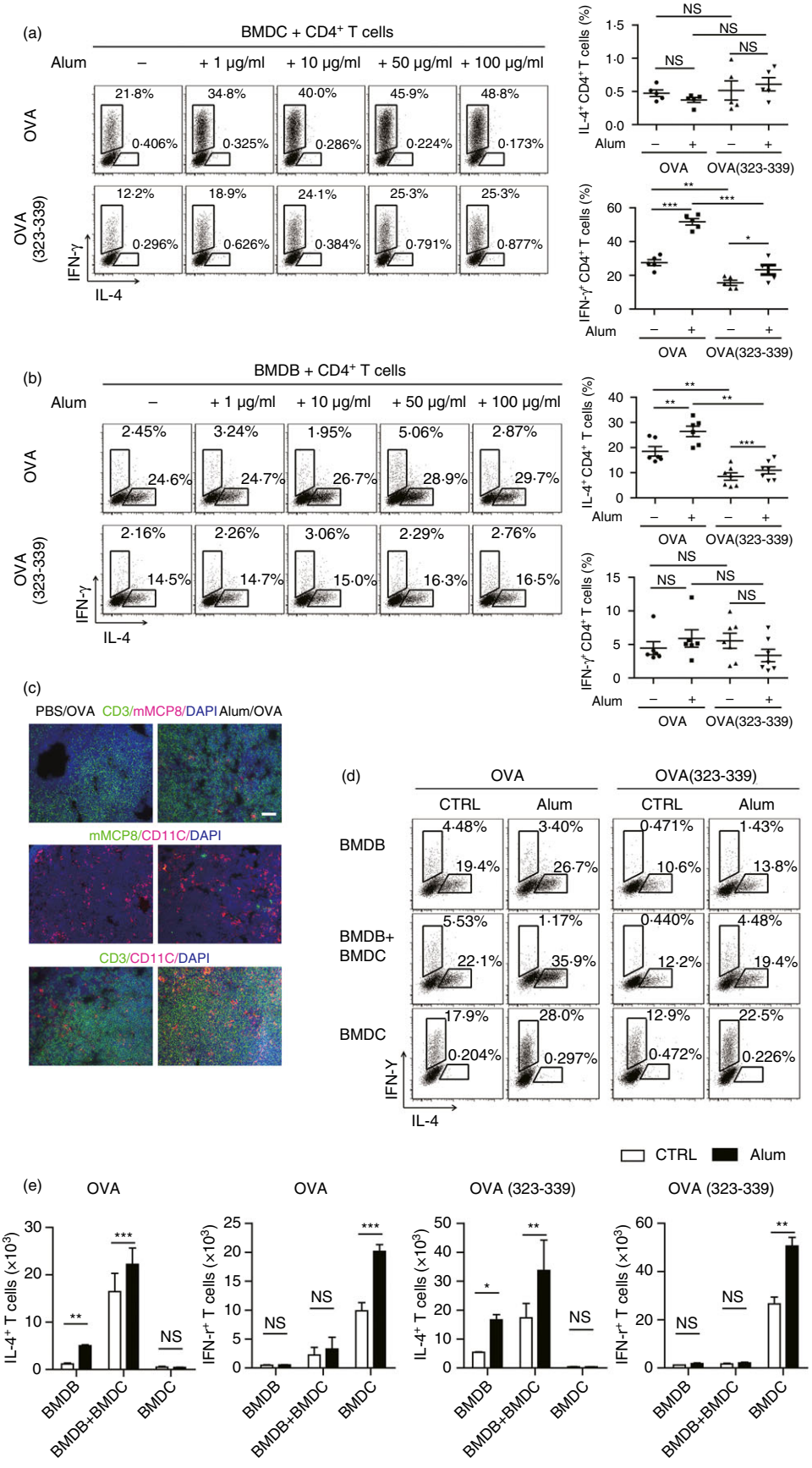

Figure 1. Alum was able to enhance bone-marrow derived basophil (BMDB) -induced T helper type 2 (Th2) differentiation *in vitro*. (a) Sorted wild-type (WT) bone-marrow-derived dendritic cells (BMDCs) ($CD11c^+$) or (b) WT bone-marrow-derived basophils (BMDBs) ($MAR1^+$ $CD49b^+$ c-kit- $CD11c^-$) pretreated with or without different doses of alum were co-cultured with DO11.10 naive $CD4^+$ T cells ($CD4^+$ $CD62L^+$ $KJ1\text{-}26^+$) at a 1 : 2 ratio in the presence of ovalbumin (OVA) protein (40 μg/ml) or OVA (323–339) (5 μg/ml). Six days later, interleukin-4 (IL-4) -producing Th2 cells and interferon-γ (IFN-γ)-producing Th1 cells were detected by intracellular cytokine staining. Numbers as shown indicated Th2 cells or Th1 cells in total live $CD4^+$ T cells. A representative ICC staining (left) and the summarized results from five (a) or six (b) independent experiments (right) are shown. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$; $***P < 0{\cdot}001$ by unpaired t-text. NS, no significant difference. (c) WT mice were immunized with 25 μg OVA in 40 μl alum adjuvant, control mice were injected with OVA in PBS via the footpad for 3 days. Basophils ($MCP8^+$), T cells ($CD3^+$) and DCs ($CD11c^+$) in draining popliteal lymph nodes (pLNs) were stained by immunofluorescence. Scale bar, 50 μm. (d) WT BMDBs, WT BMDCs or a combination of BMDBs and BMDCs pretreated with or without alum were co-cultured with DO11.10 naive $CD4^+$ T cells ($CD4^+$ $CD62L^+$ $KJ1\text{-}26^+$) in the presence of OVA protein or OVA (323–339). Six days later, IL-4-producing Th2 cells and IFN-γ-producing Th1 cells were detected by intracellular cytokine staining. A representative ICC staining from three separate experiments is shown. (e) The total number of IL-4-producing Th2 cells and IFN-γ-producing Th1 cells gated in live cells from three experiments in the presence of OVA or peptide is shown. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$; $***P < 0{\cdot}001$ by Two-way analysis of variance. NS, no significant difference.

illustrated in the relevant figure legends with significance as: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ and $***P < 0{\cdot}001$.

Results

Alum enhanced the ability of murine basophils not dendritic cells to induce Th2 differentiation

As reported previously,[9,21] IgE production promoted by alum was largely diminished when IL-4-KO mice were immunized with OVA adsorbed to alum (see Supplementary material, Fig. S1a). In contrast, the IgG1 response, although significantly reduced compared with WT mice, was still present in IL-4-KO mice (see Supplementary material, Fig. S1a). Furthermore, IL-5-producing Th2 cells in spleen were not altered in challenged IL-4-KO mice (see Supplementary material, Fig. S1b). Therefore, an IL-4-independent mechanism is indicated in the alum-promoted Th2 response.

To uncover mechanisms responsible, we investigated the type of APCs that can be directly affected by alum exposure. We initially examined if DCs, the most efficient APCs that can activate and differentiate naive T cells, are sensitive to alum stimulation for construction of Th2 cell development. As shown in Fig. 1(a), treatment of BMDCs by alum did not result in any IL-4-producing Th2 cells in the DC-$CD4^+$ T-cell co-culturing experiment, whereas interferon-γ-producing Th1 cells were significantly enhanced when DCs were treated with alum (Fig. 1a). This indicated that alum cannot steer DCs from polarizing Th1 cell development. A recent study revealed an important role played by basophils for induction of alum-promoted Th2 immune response,[13] and has prompted us to question whether alum could act on basophils directly to boost their Th2-promoting activities.

To address this, BMDBs were co-cultured with OVA peptide-specific naive $CD4^+$ T cells from DO11.10 mice. We found that BMDBs mainly polarized naive $CD4^+$ T cells into Th2 cells with little induction of Th1 cells whether OVA protein or peptide was supplemented (Fig. 1b). When BMDBs were treated with alum, Th2-polarization capacities of BMDBs were further enhanced with increased doses of alum being applied (Fig. 1b). Notably, high or low ratios between basophils and $CD4^+$ T cells examined all displayed enhanced Th2-promoting effects by alum (see Supplementary material, Fig. S2), and the ability of alum to enhance the capacity of basophils to skew Th2 cells was not related to different induction of $CD4^+$ T-cell proliferation (see Supplementary material, Fig. S3). Therefore, in contrast to DCs, basophils are intrinsically potent Th2-promoting APCs and this activity can be further enhanced by alum.

When fold increase values in interferon-γ-producing Th1 cells from BMDCs or in IL-4-producing Th2 cells from BMDBs induced by alum in Fig. 1(a,b) were calculated and compared, higher levels of Th1 fold increase induced by alum-treated DCs were observed (see Supplementary material, Fig. S4). This made us question which type of Th cell development would dominate when both DCs and basophils are present upon alum exposure. This becomes especially significant when more basophils were migrated into draining lymph nodes and co-localized with DCs and T cells at T-cell area 3 days after alum/OVA immunization (Fig. 1c). When Th polarization profiles were studied after naive $CD4^+$ T cells were co-cultured with BMDBs plus BMDCs with or without alum, we found that Th2 cell development was enhanced with greatly depressed Th1 cell development when both basophils and DCs were present (Fig. 1d). Furthermore, the production of Th2 cells was further enhanced when alum was added (Fig. 1d), which was especially prominent when total numbers of induced Th2 cells were calculated (Fig. 1e). When we further question if the collaboration of basophils and DCs is mediated by cell–cell contact or by soluble factors secreted by basophils, we found that supernatants from 18-hr-cultured basophils were able to confer similar levels of Th2-promoting activities by DCs compared with that induced by basophils being directly contacted with DCs (see Supplementary material, Fig. S5 versus Fig. 1d). Taken together, these data demonstrated that Th2-inducing milieu (basophils) plays a more dominant role over the Th1-inducing milieu (BMDCs) as reported.[17]

Alum enhanced the capacities of basophils to polarize Th2 cells via an IL-4-independent pathway

As indicated by our *in vivo* data (see Supplementary material, Fig. S1), an IL-4-independent mechanism should exist in Th2-promoting effects by alum. We then questioned whether or not IL-4 is involved in boosted Th2 polarization activities by alum-treated basophils found *in vitro*. Large amounts of both IL-4 mRNA and

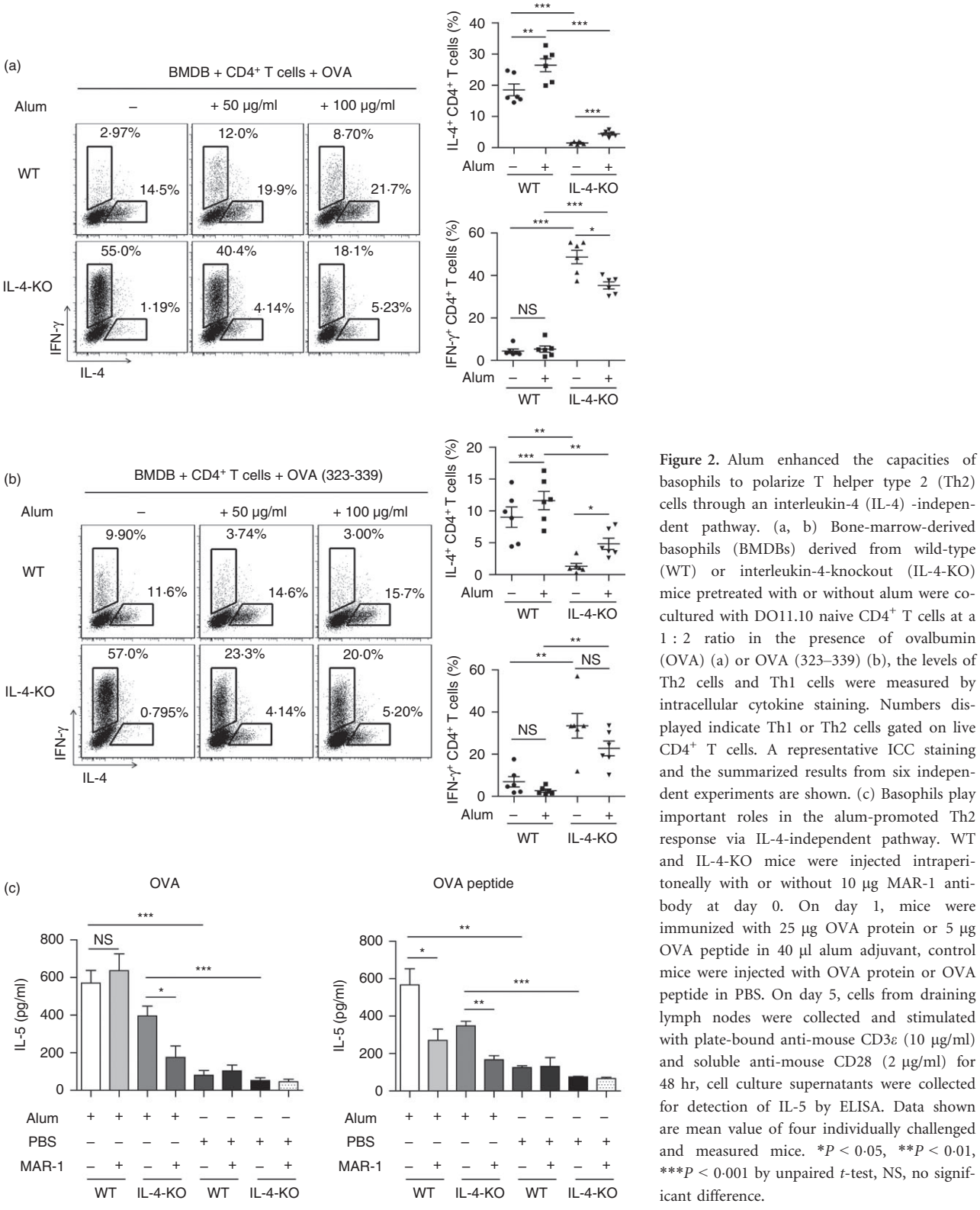

Figure 2. Alum enhanced the capacities of basophils to polarize T helper type 2 (Th2) cells through an interleukin-4 (IL-4) -independent pathway. (a, b) Bone-marrow-derived basophils (BMDBs) derived from wild-type (WT) or interleukin-4-knockout (IL-4-KO) mice pretreated with or without alum were co-cultured with DO11.10 naive $CD4^+$ T cells at a 1 : 2 ratio in the presence of ovalbumin (OVA) (a) or OVA (323–339) (b), the levels of Th2 cells and Th1 cells were measured by intracellular cytokine staining. Numbers displayed indicate Th1 or Th2 cells gated on live $CD4^+$ T cells. A representative ICC staining and the summarized results from six independent experiments are shown. (c) Basophils play important roles in the alum-promoted Th2 response via IL-4-independent pathway. WT and IL-4-KO mice were injected intraperitoneally with or without 10 μg MAR-1 antibody at day 0. On day 1, mice were immunized with 25 μg OVA protein or 5 μg OVA peptide in 40 μl alum adjuvant, control mice were injected with OVA protein or OVA peptide in PBS. On day 5, cells from draining lymph nodes were collected and stimulated with plate-bound anti-mouse CD3ε (10 μg/ml) and soluble anti-mouse CD28 (2 μg/ml) for 48 hr, cell culture supernatants were collected for detection of IL-5 by ELISA. Data shown are mean value of four individually challenged and measured mice. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$ by unpaired *t*-test, NS, no significant difference.

IL-4 protein were constitutively expressed in BMDBs. Alum treatment did not enhance their expression levels further (see Supplementary material, Fig. S6). Basophils from IL-4-KO animals were then used to determine the role of IL-4 in Th2-enhancing effects by alum. As reported elsewhere,[14–16] intrinsic Th2-inducing activities by basophils were largely IL-4-dependent because induction of Th2 cells was almost completely abrogated when basophils from IL-4-KO mice were used in co-culturing experiments in the presence of either OVA protein (Fig. 2a) or OVA peptide (Fig. 2b). Induction of Th1 cells by basophils from IL-4-KO mice was greatly increased, probably due to lowered levels of Th2 cells (first panel on Fig. 2a,b). When a similar experiment was performed on alum-treated basophils from IL-4-KO mice, Th2 cells were still induced with levels similar to the increased levels induced by alum treatment of WT basophils (Fig. 2a,b). Therefore, an IL-4-independent mechanism may constitute an important part in conferring boosted Th2-promoting activities seen on alum-treated basophils, at least *in vitro*.

To further examine the role played by basophils in promoting alum-induced Th2 responses in an IL-4-independent scenario, IL-5 production was examined in WT and IL-4-KO mice that were immunized with alum/OVA or alum/OVA peptide 24 hr after basophil depletion by intraperitoneal injection of antibody MAR-1 to mouse FcεRIα (see Supplementary material, Fig. S7).[8] PBS/OVA or PBS/OVA peptide immunization was used as control. As shown in Fig. 2(c), a significant amount of IL-5 was induced when alum was administered together with antigens both in WT and in IL-4-KO mice compared with PBS control. Although IL-5 contents were reduced in immunized IL-4-KO mice, the remaining IL-5 produced independent of IL-4 was further reduced upon basophil depletion whether immunized with alum/OVA or with alum/OVA peptide (Fig. 2c). It indicated that basophils can enhance alum-elicited Th2 responses through an IL-4-independent pathway. Furthermore, depletion of basophils also resulted in reduced IL-5 production in IL-4-intact mice if OVA peptides were injected together with alum but not when OVA proteins were used. Hence, we concluded that basophils contribute to an alum-stimulated IL-4-independent Th2 response, a pathway that may function under some situations when IL-4 is present. Meanwhile, the IL-4-dependent pathway should still be the dominant mechanism.

Soluble factors were ascribed to IL-4-independent Th2 polarization activities possessed by alum-treated basophils

Our data so far predicted that alum could enhance basophils to induce Th2 differentiation through an IL-4-independent pathway. We next tested if non-IL-4 soluble factors produced by alum-treated basophils are involved. To address this question, supernatants were generated by stimulating basophils from IL-4-KO mice with alum for 18 hr. High-speed centrifugation was performed to remove cells and alum. Absence of alum in these cell-free supernatants was verified by its incapability of activation of NLRP3 in lipopolysaccharide-primed macrophages (see Supplementary material, Fig. S8). These supernatants were then examined for their ability to allow untreated IL-4-KO basophils to gain alum-boosted Th2-inducing activities.

As shown in Fig. 3(a,b), whether OVA protein or peptide was added, non-IL-4-containing supernatants from alum-treated basophils were able to regain the Th2-inducing activities lost by untreated IL-4-KO basophils when they were co-cultured with DO11.10 $CD4^+$ T cells. To confirm the above findings, skewed $CD4^+$ T cells were re-stimulated with plate-bound anti-mouse CD3ε to measure either GATA-3 mRNA, the signature transcriptional factor for Th2 cells,[22] or IL-4 protein in supernatants. As was found by intracellular cytokine staining for IL-4 (Fig. 3a,b), IL-4 secretion as well as GATA-3 mRNA by reactivated $CD4^+$ T cells were both enhanced by alum-treated basophil supernatants (Fig. 3c). Furthermore, the Th2 induction levels induced by supernatants generated from IL-4-KO alum-treated basophils were comparable to the levels induced by alum-treated basophils from IL-4-KO mice (Fig. 3d). These data firmly established that alum can enhance Th2-promoting activity of basophils through non-IL-4 soluble factors.

We next examined possible non-IL-4 soluble molecules responsible. IL-25[23] and TSLP,[8,24] two major non-T-cell-derived Th2-promoting cytokines, were found to be produced by basophils.[8,23] To exclude possible influences of IL-4 on the gene transcription of these two cytokines, RNAs were extracted from IL-4-KO basophils with or without alum treatment and measured for mRNA content of IL-25 and TSLP by real−time PCR. As shown in Fig. 3(e), TSLP and IL-25 mRNA transcripts were all induced in IL-4-KO basophils by alum stimulation compared with unstimulated basophils. Additionally, the levels of TSLP and IL-25 mRNA induced in the absence of IL-4 were similar to those induced in the presence of IL-4 (Fig. 3e), which suggested that basophils may use these two cytokines to achieve their Th2-promoting activities in an IL-4-independent fashion. To further assess if these two cytokines actually contribute to non-IL-4-controlled Th2 differentiation by basophils following alum exposure, supernatants were pretreated with neutralizing antibodies for IL-25 or TSLP or a combination of both antibodies before being added respectively into an IL-4-KO basophils and DO11.10 naive $CD4^+$ T cells co-culturing system. Addition of anti-TSLP or anti-IL-25 antibodies only or a combination of both antibodies can decrease the levels of Th2 cells elicited by alum (Fig. 3f). Taking these findings together, TSLP or IL-25

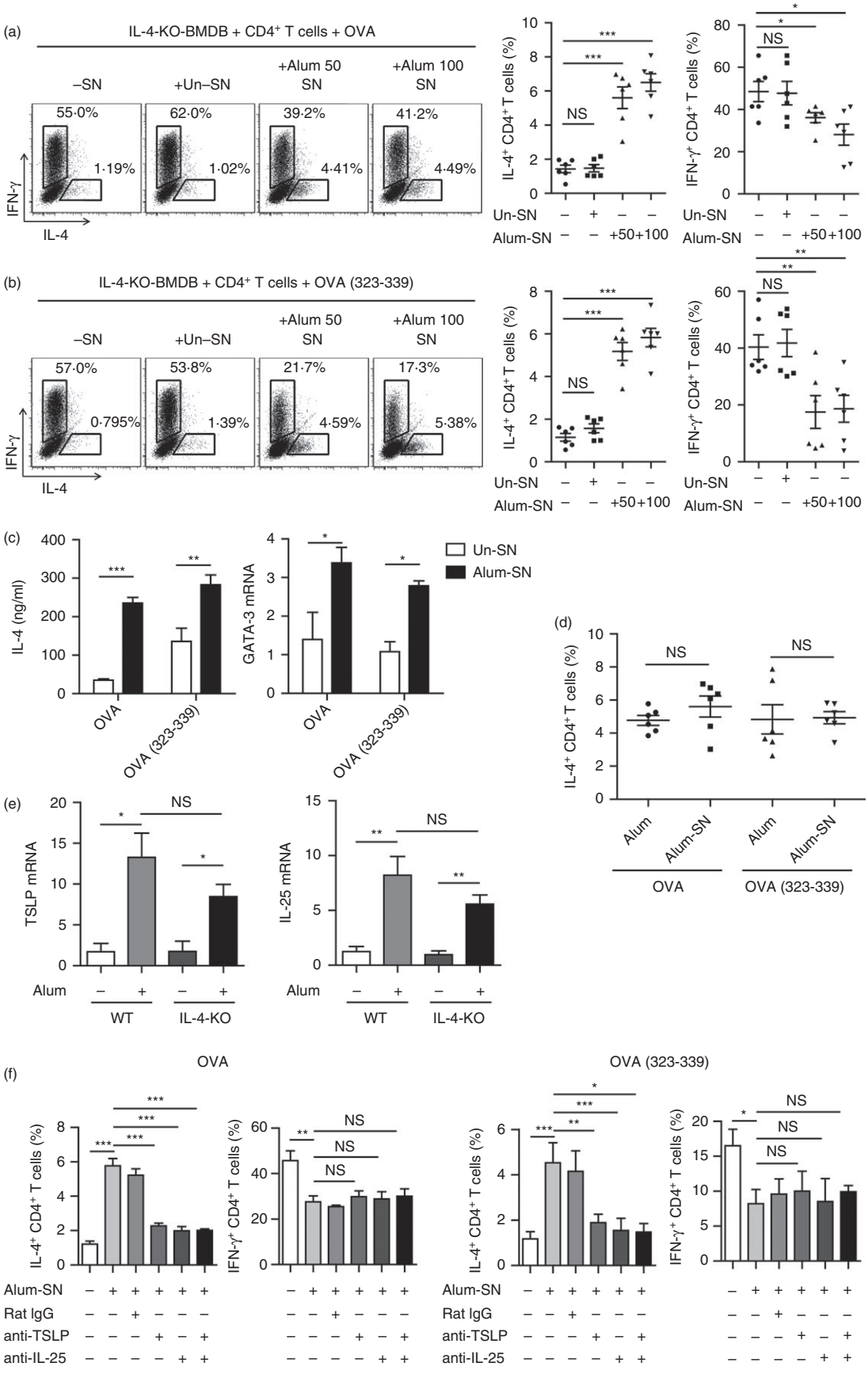

Figure 3. Soluble factors contributed to interleukin-4 (IL-4) -independent T helper type 2 (Th2) polarization induced by alum-treated basophils. (a,b) Bone-marrow-derived basophils (BMDBs) from interleukin-4-knockout (IL-4-KO) mice were co-cultured with DO11.10 $CD4^+$ T cells with or without addition of cell- and alum-free supernatants (SN) derived from unstimulated (Un-SN) or alum-treated (Alum-SN) IL-4-KO BMDBs in the presence of ovalbumin (OVA) (a), or OVA (323–339) (b) for 6 days. The supernatants were added to BMDBs and the $CD4^+$ T-cell co-culturing system at half of the total culture volume. One representative flow cytometry graph of Th1 and Th2 cells (left) and summarized data from six experiments (right) are shown. (c) After 6 days in experiments a and b, $CD4^+$ T cells were re-stimulated with plate-bound anti-mouse CD3ε (10 μg/ml) for 18 hr. Cell-free supernatants were collected for detection of IL-4 protein by ELISA (left panel) and T cells were collected for the measurement of mRNA content of GATA-3 by real-time PCR (right panel), which levels were calculated by value of $2^{-\Delta\Delta Ct}$ as shown in the Materials and methods. (d) Comparison between Th2 levels from IL-4-KO a-basophils (Alum) and from supernatants generated from IL-4-KO a-basophils (Alum-SN) summarized from six independent experiments is shown. (e) Real-time PCR analysis of mRNA expression of IL-25 and thymic stromal lymphopoietin (TSLP) in unstimulated or alum-treated wild-type (WT) or IL-4-KO basophils, expression levels were calculated by the value of $2^{-\Delta\Delta Ct}$ as shown in the Materials and methods. Bar graphs represented the mean + SEM from three independent experiments. (f) Cell-free supernatants derived from alum-treated IL-4-KO basophils (Alum-SN) were pre-incubated with neutralizing antibodies for anti-IL-25 (10 μg/ml), anti-TSLP (10 μg/ml), anti-IL-25 plus anti-TSLP or isotype-matched control antibody-Rat IgG (10 μg/ml) for 1 hr before adding to the IL-4-KO basophils and $CD4^+$ T cells co-culturing system in the presence of OVA (left panel) or OVA (323–339) (right panel). Summarized results from three or four independent experiments are shown. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$; $***P < 0{\cdot}001$ by unpaired *t*-test. NS, no significant difference.

can contribute to non-IL-4-controlled Th2 differentiation activities by alum on basophils.

The enhanced capacities of basophils to promote Th2 cells induced by alum were NLRP3-independent

NLRP3, a member of the NLRs, has so far been recognized as the only important intracellular sensor molecule for alum, which is essential for alum-induced inflammasome activation in professional APCs such as macrophages and DCs. Furthermore, some reports showed that NLRP3 is critical for alum's adjuvant effect *in vivo*.[25] Here, we questioned if the NLRP3 molecule plays an important role in alum-promoted Th2 differentiation on basophils.

We first assessed NLRP3 expression in murine basophils. NLRP3 mRNA transcript was detected in basophils with similar levels to that on macrophages and DCs (Fig. 4a). More significantly, alum, together with lipopolysaccharide, was also able to activate basophils to produce mature IL-1β and the active form of caspase-1 in NLRP3-dependent fashion (Fig. 4b), like alum in macrophages.[26] Therefore, NLRP3 is present and functional in murine basophils regarding the NLRP3 inflammasome activation in response to alum.

Next we tested whether the induction of Th2 cell development by alum-treated basophils is dependent on NLRP3. As shown in Fig. 4(c,d), unstimulated basophils from NLRP3-KO mice were still able to activate naive $CD4^+$ T cells and induce strong Th2 cells with slightly, but not significantly, reduced levels compared with WT mice. More importantly, the enhancing effects of alum on Th2 differentiation were similar between basophils obtained from WT and NLRP3-KO mice. Hence, our results indicated for the first time that NLRP3 is present in murine basophils, but NLRP3 is dispensable for alum-enhanced Th2 polarization activities by murine basophils.

Discussion

As the earliest approved adjuvant used in humans, alum is still widely administered to make many vaccines work successfully. Due to its adjuvant effects, which are mainly reflected in the boosted Th2-directed antibody response, alum is regarded as a Th2-promoting agent.[9,21] Unlike other characterized Th2-promoting agents like cholera toxin,[27] prostaglandin E_2[28] or egg antigens from worms,[29,30] whether or not alum can play any direct modulatory effects on DCs is not certain.[11,12] Our result found no direct Th2-enhancing effects on DCs by alum treatment, which is similar to previous reports.[12] Therefore, the nature of APCs, which can be modified by alum directly to instruct Th2 differentiation, if there is any, remain elusive.

Many kinds of innate cells are all found at injected sites following alum-adsorbed antigen immunization. Eosinophils, neutrophils, monocytes and DCs are all present in the peritoneal cavity following intraperitoneal injection of alum,[10] whereas basophils can be found at the skin injection site.[13] Among them, monocyte-derived DCs and basophils have both been shown to be important in adjuvant activities of alum, as immunized mice deficient for these cells display defects in alum boosted immune responses.[10,13] Our result found no direct Th2-enhancing effects but rather Th1-enhancing effects on DCs by alum treatment, so we focused on the effects of alum on basophils to boost the Th2 response and associated mechanisms. Our result demonstrated that alum is able to directly up-regulate the Th2-promoting activities by basophils as well as Th1-promoting activities by DCs. Importantly, it is the Th2-inducing activities that predominate when both basophils and DCs are present, which may explain how Th2-associated adjuvanticity constantly predominates following administration of alum *in vivo*. Furthermore, the levels of Th2 cells are greatly enhanced

when DCs co-exist with basophils, which may be the reason that DCs are also shown to be required for Th2-associated adjuvanticity by alum[10] even though alum apparently strengthens Th1-skewing activities on DCs. These findings become particularly relevant *in vivo* in view of our findings that presence of alum in an immunization regimen is able to drive more basophils to migrate or infiltrate into draining lymph nodes in T-cell areas that are co-localized with both DCs and T cells.

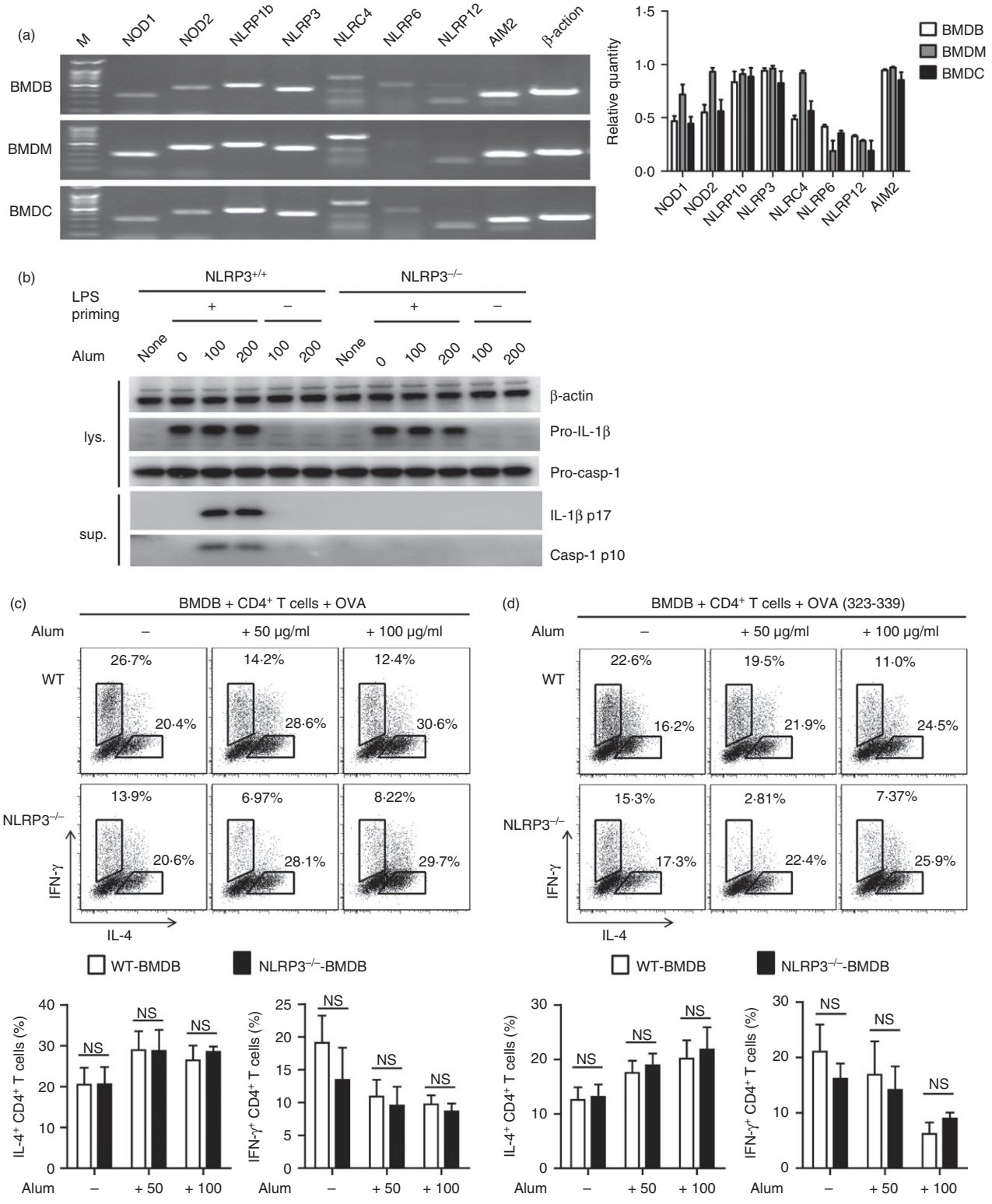

Figure 4. The enhanced capacities of basophils to promote T helper type 2 (Th2) cells induced by alum were NLRP3-independent. (a) Expression of nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) mRNA in bone-marrow-derived basophils (BMDBs) ($n = 3$), bone-marrow-derived macrophages (BMDMs) ($n = 3$) and bone-marrow-derived dendritic cells (BMDCs) ($n = 3$) determined by RT-PCR. Calculated levels from densitometry analysis are shown by bars as mean + SEM. (b) BMDBs from wild-type (WT) and NLRP3-knockout (KO) mice were primed with or without lipopolysaccharide (LPS; 100 ng/ml) for 3 hr followed by stimulation for an additional 6 hr with different doses of alum (μg/ml). Production of mature interleukin-1β (IL-1β) (p17) and active caspase-1 (p10) was measured by Western blot in supernatants (sup.) and pro-IL-1β, pro-caspase-1 and β-actin in cell lysates (lys.). Data were from one of at least three independent experiments. (c) BMDBs from WT or NLRP3-KO mice pretreated with or without indicated doses of alum were co-cultured with OT-II $CD4^+$ T cells at 1 : 2 ratio in the presence of OVA (c) or OVA (323–339) (d) for 6 days. Th1 or Th2 polarization profiles with summarized results from three independent experiments are shown. NS, no significant difference.

Characterized by production of IL-4, IL-5 and others, Th2 differentiation has been known to be driven mainly by IL-4 since its discovery.[31] The observations made by us (see Supplementary material, Fig. S1) and others[9,21] certainly indicated that both IL-4-dependent and IL-4-independent mechanisms are implicated in alum-promoted Th2 responses. The participation of basophils *in vivo* in orchestrating alum-promoted Th2 responses is supported by genetic ablation of basophils[13] as well as by our own data using antibodies against CD200R3 to deplete basophils, a surface molecule rarely expressed by Th2-skewing FcεRI-positive DC subsets (our and House dust mite and data not shown).[32] Although basophils are known to be good IL-4-producing cells,[14–16] our results indicate that basophils can enhance Th2-inducing ability by alum through IL-4-independent mechanisms both *in vitro* and *in vivo*. A peculiar finding is that the basophil-mediated IL-4-independent pathway is apparent only when IL-4 is not around, regardless of whether OVA proteins or peptides were used as immunogens (Fig. 2c). When IL-4 is intact, basophil depletion resulted in decreased levels of Th2 cells only when animals were immunized with OVA peptides, similar to findings by genetic ablation of basophils.[13] These data suggested that the IL-4-dependent pathway should still be the dominant mechanism. Under some normal situations, basophils may need to be placed in close proximity to T cells and possibly to DCs as well so as to exert their IL-4-independent Th2-promoting effects.

Interleukin-4-independent mechanisms for Th2 development have been observed in helminth infection or allergens.[33] In these studies, production of IL-25, IL-33 and TSLP have all been shown to be involved. Non-T cells including epithelial cells,[17,34] basophils,[8,23] eosinophils[23] and mast cells[35,36] may all take part in orchestrating Th2 responses through production of these cytokines, as these innate sources of non-IL-4 cytokines are able to promote Th2 responses either by directly acting on T cells or by indirectly facilitating DC2 activities.[37,38] We found in this study that alum is able to enhance the production of IL-25 and TSLP independent of IL-4, but not production of IL-4 in treated basophils. More significantly, neutralization of the activities of either of these two cytokines is able to abrogate Th2-enhancing effects by supernatants derived from alum-treated IL-4-KO basophils with no synergistic effects being observed. All of these indicated, at least *in vitro*, that stimulation of both the IL-25 and TSLP pathways together is required for IL-4-independent basophil-mediated Th2 polarization promoted by alum. Furthermore, the IL-25 and TSLP generated by alum-stimulated basophils should conceivably favour DCs to perform Th2-promoting activities.[38] However, neutralization antibody against IL-25 or TSLP cannot block the effects of alum-promoted Th2 polarization on WT basophils (see Supplementary material, Fig. S9), which is consistent with the theme that the IL-4-dependent pathway is predominant. It awaits to be clarified whether IL-4-independent production of IL-25 or TSLP by basophils contributes directly to alum-induced Th2-promoting adjuvant activities *in vivo*.

NLRP3 is the only well-characterized intracellular sentinel pattern recognition receptor for sensing the presence of alum. Early reports on the discovery of NLRP3 inflammasome have shown that alum can activate caspase-1 in professional APCs, resulting in the release of mature IL-1β, IL-18 and IL-33.[20,25,26,39] Our result for the first time demonstrated that murine bone-marrow-derived basophils also constitutively express NLRP3 and alum can activate NLRP3 inflammasomes in basophils as well as in professional APCs. Alum is thought to activate NLRP3 inflammasome by itself[20] or through induction of other kinds of NLRP3-activating molecules like uric acid.[10] However, the role played by NLRP3 in the alum-boosted Th2-directed humoral response appears to be controversial.[26,39,40] Reports have shown that vaccination with antigen and alum requires NLRP3, ASC and caspase-1 to induce antigen-specific IgG1 production.[26,39] But other studies reported dispensable roles played by NLRP3 for alum-mediated adjuvant activity.[40] In fact, some NLRP3-irrelevant mechanisms, like induction of cell death[41] and lipid membrane configuration change,[42] have been shown to be essential for alum adjuvant activities. Our data demonstrated that NLRP3 is dispensable in alum-enhanced Th2-promoting activities by basophils.

In summary, the data presented in this work demonstrated that alum enhances basophils but not DCs to

promote Th2 differentiation via an IL-4- and NLRP3-independent pathway. Increased production of TSLP and IL-25 by alum-treated basophils may contribute to the observed alum-promoted Th2 cells by basophils. Our study indicated important roles played by non-IL-4 cytokines released by basophils in orchestrating Th2-directed immune responses by alum.

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Authorship contributions

Contributions: F.-J.H., L.Y. and X.-P.C. designed research; F.-J.H., Y.-L.M., R.-Y.T., W.-C.G. and J.L. performed research; F.-J.H., and C.-C.X. analysed data; F.-J.H., L.Y. and X.-P.C. wrote the paper. All authors have read and approved the final manuscript.

Disclosures

The authors declare no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Induction of T helper type 2 (Th2) cells and Th2-directed IgG1 response by alum can be via interleukin-4-independent pathway.

Figure S2. High or low ratios between basophils and $CD4^+$ T cells examined all displayed enhanced T helper type 2-promoting effects by alum

Figure S3. The ability of alum to enhance the capacity of basophils to skew T helper type 2 cells was not related to different induction of $CD4^+$ T cells proliferation.

Figure S4. Increased levels of T helper type 1 cells induced by alum-treated dendritic cells was higher compared with the increased levels of T helper type 2 cells on alum-treated basophils.

Figure S5. The T helper type 2-promoting bone-marrow-derived dendritic cells facilitated by basophils can be mediated by soluble factors secreted by bone-marrow-derived basophils.

Figure S6. Alum treatment did not enhance expression levels of interleukin-4 mRNA and interleukin-4 protein.

Figure S7. Basophils were depleted by intraperitoneal injection of MAR-1 antibody.

Figure S8. High-speed centrifugation of supernatants largely removed alum.

Figure S9. T helper type 2 cells elicited by alum-treated wild-type basophils were not largely reduced whether blocking thymic stromal lymphopoietin or interleukin-25 or both by antibodies.

Table S1. Primer used in real-time RT-PCR.

Table S2. Primer used in RT-PCR for NLRs.